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Diaporthe camptothecicola sp. nov. on *Camptotheca acuminata* in China

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Abstract—A novel species *Diaporthe camptothecicola* is described and illustrated from cankers on branches of *Camptotheca acuminata* (*Cornaceae*) collected at Nanjing Agricultural University, Jiangsu Province, China. Recognition of this new species is supported by both morphology and phylogeny. The alpha conidia are hyaline, aseptate, oblong (6.2 × 1.6 µm), and inconspicuously biguttulate, similar to most species of *Diaporthe*. The beta conidia are hyaline, aseptate, filiform to hamate, 25.1 × 1.0 µm. Multi-locus phylogenetic analysis based on ITS, CAL, HIS, TEF1-α, and TUB2 gene regions suggests the new species belongs to *Diaporthe*.

Key words—ascomycete, *Diaporthales*, dieback, systematics, taxonomy

Introduction

Members of *Diaporthe* are plant pathogens, endophytes, and saprobes with extensive host ranges and wide geographic distributions (Wehmeyer 1933; Uecker 1988; Crous & Groenwald 2005, Rossman et al. 2007; Udayanga et al. 2011, 2012a, b, 2014a, b, 2015; Huang et al. 2013, 2015; Gao et al. 2014, 2015, 2016; Fan et al. 2015; Du et al. 2016). This genus is characterized by ostiolate conidiomata with elongate, cylindrical phialides and may produce three types conidia, of which alpha and beta conidia are produced frequently (Udayanga et al. 2011). The sexual morph is characterized by immersed ascomata and

Table 1. *Diaporthe* isolates and sequences used in the phylogenetic analysis

Species	Isolate	Host	Location	GenBank accession numbers				
				ITS	CAL	HIS	TEF1-α	TUB2
D. alleghaniensis	CBS 495.72^{T}	*Betula alleghaniensis*	Canada	KC343007	KC343249	KC343491	KC343733	KC343975
D. alnea	CBS 146.46^{T}	*Alnus* sp.	Netherlands	KC343008	KC343250	KC343492	KC343734	KC343976
	CBS 159.47	*Alnus* sp.	Netherlands	KC343009	KC343251	KC343493	KC343735	KC343977
	LCM22b.02a	*Alnus* sp.	USA	KJ210535	KJ435020	KJ420883	KJ210557	KJ420825
	LCM22b.02b	*Alnus* sp.	USA	KJ210536	KJ435021	KJ420884	KJ210558	KJ420826
D. apiculata	LC3418^{T}	*Camellia sinensis*	China	KP267896	—	—	KP267970	KP293476
D. bicincta	CBS 121004^{T}	*Juglans* sp.	USA	KC343134	KC343376	KC343618	KC343860	KC344102
D. biguttusis	CGMCC 3.17081^{T}	*Lithocarpus glaber*	China	KF576282	—	—	KF576257	KF576306
	CGMCC 3.17082	*Lithocarpus glaber*	China	KF576283	—	—	KF576258	KF576307
D. camptothecicola	**CFCC 51632^{T}**	***Camptotheca acuminata***	**China**	**KY203726**	**KY228877**	**KY228881**	**KY228887**	**KY228893**
	CFCC 51633	***Camptotheca acuminata***	**China**	**KY203727**	**KY228878**	**KY228882**	**KY228888**	**KY228894**
D. celastrina	CBS 139.27^{T}	*Celastrus* sp.	USA	KC343047	KC343289	KC343531	KC343773	KC344015
D. citrichinensis	ZJUD 34A^{T}	*Citrus* sp.	China	JQ954648	KC357494	—	JQ954666	—
	ZJUD 34B	*Citrus* sp.	China	JQ954649	KC357495	—	JQ954667	KC357461
D. ellipicola	CGMCC 3.17084^{T}	*Lithocarpus glaber*	China	KF576270	—	—	KF576245	KF576291
	CGMCC 3.17085	*Lithocarpus glaber*	China	KF576271	—	—	KF576246	KF576292
D. eres	AR5193^{T}	*Ulmus* sp.	Germany	KJ210529	KJ434999	KJ420850	KJ210550	KJ420799
	FAU483	*Malus* sp.	Netherlands	KJ210537	KJ435022	JQ807422	KJ420874	KJ420827
	DNA001A	*Daphne laureola*	France	KJ210516	KJ434994	KJ210540	KJ420831	KJ420781

D. eres [continued]	DNA001B	*Daphne laureola*	France	KJ210517	KJ434995	KJ210541	KJ420832	KJ420782
	AR3519	*Corylus avellana*	Austria	KJ210523	KJ435008	KJ210547	KJ420839	KJ420789
	AR3723	*Rubus fruticosus*	Austria	JQ807428	KJ435024	JQ807354	KJ420843	KJ420793
	AR3560	*Viburnum* sp.	Austria	KJ420795	KJ435011	JQ807351	KJ420846	KJ420795
	AR5223	*Acer negundo*	Germany	KJ210528	KJ435000	KJ210549	KJ420845	KJ420830
D. gardeniae	CBS 288.56	*Gardenia florida*	Italy	KC343113	KC343355	KC343597	KC343839	KC344081
D. helicis	AR5211^{T}	*Hedera helix*	France	KJ210538	KJ435043	KJ420875	KJ210559	KJ420828
D. longicicola	CGMCC 3.17089^{T}	*Lithocarpus glaber*	China	KF576267	—	—	KF576242	KF576291
	CGMCC 3.17090	*Lithocarpus glaber*	China	KF576268	—	—	KF576243	KF576292
D. mahothocarpi	CGMCC 3.15181^{T}	*Lithocarpus glaber*	China	KC153096	—	—	KC153087	KF576312
D. maritima	DAOMC 250563	*Picea rubens*	Canada	KU552025	—	—	KU552023	KU574615
D. neilliae	CBS 144.27^{T}	*Spiraea* sp.	USA	KC343144	KC343386	KC343628	KC343870	KC344112
D. nobilis	CBS 113470	*Castanea sativa*	Korea	KC343146	KC343388	KC343630	KC343872	KC344114
D. nomurai	CBS 157.29	*Morus* sp.	Japan	KC343154	KC343396	KC343638	KC343880	KC344122
D. oraccinii	LC3166^{T}	*Camellia sinensis*	China	KP267863	—	KP293517	KP267937	KP293443
D. penetritea	LC3353^{T}	*Camellia sinensis*	China	KP714505	—	KP714493	KP714517	KP714529
D. pulla	CBS 338.89^{T}	*Hedera helix*	Yugoslavia	KC343152	KC343394	KC343636	KC343878	KC344120
D. subclavata	ICMP 20663^{T}	*Citrus unshiu*	China	KJ490587	—	KJ490529	KJ490466	KJ490408
D. vaccinii	CBS 160.32^{T}	*Oxycoccus macrocarpos*	USA	KC343228	KC343470	KC343712	KC343954	KC344196
	DF5042	*Vaccinium corymbosum*	USA	AF317570	KC849457	KJ420877	JQ807380	KC843225
D. citri	AR3405^{T}	*Citrus* sp.	USA	KC843311	KC843157	KJ420881	KC843071	KC843187

New species are bold.

erumpent pseudostroma with more or less elongated perithecial necks; asci are unitunicate, clavate to clavate-cylindrical; and ascospores are biseriate to uniseriate in the ascus, septate, hyaline, and sometimes with appendages (Wehmeyer 1933, Muntañola-Cvetković et al. 1981).

During collecting trips in Jiangsu Province, China, fresh specimens were collected from symptomatic cankered branches of *Camptotheca acuminata*. A fungal specimen was found with characters fitting the genus *Diaporthe*. Because species of *Diaporthe* cannot easily be distinguished morphologically, a phylogenetic analysis were carried out based on ITS, CAL, HIS, TEF1-α, and TUB2 gene regions. This analysis determined that this species belongs in *Diaporthe* and is distinct from all other currently described and sequenced species in the *D. eres* species complex.

Materials & methods

Samples and isolates

The isolates were obtained from two specimens by single spore isolation following the methods of Fan et al. (2014). Single germinating conidia were removed and plated onto fresh PDA plates after incubation at 25 °C for up to 24 h. Specimens and isolates of the fungus are deposited in the Museum of Beijing Forestry University, Beijing, China (BJFC). Axenic cultures are maintained in the China Forestry Culture Collection Center, Beijing, China (CFCC).

Morphological studies

Observations of morphological features of the fruiting bodies produced on infected plant tissues were supplemented by cultural characteristics. Vertical and horizontal sections were cut through the fruiting bodies by hand using a double-edge blade. The morphology of the fruiting bodies including size of conidiomata and locules, size and shape of conidiophores and conidia was examined under a Leica DM 2500 compound microscope. More than 20 fruiting bodies were sectioned, and 50 spores were selected randomly for measurement using a Leica LM/DM 2500 compound microscope. Cultural characteristics (e.g., colony color, texture, arrangement of the conidiomata) of isolates incubated on PDA in the dark at 25 °C were observed and recorded at 3, 7, and 30 days.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from colonies grown on PDA with cellophane using a modified CTAB method (Doyle & Doyle 1990). DNA were estimated by electrophoresis in 1% agarose gels, and the quality was measured by NanoDrop™ 2000 (Thermo, USA) according to the user's manual (Desjardins et al. 2009). The internal transcribed spacer (ITS) region was amplified with primers ITS1 and ITS4 (White et al. 1990); the CAL region with primers CAL-228F and CAL-737R (Carbone & Kohn 1999); the HIS region with primers CYLH4F (Crous et al. 2004) and H3-1b (Glass &

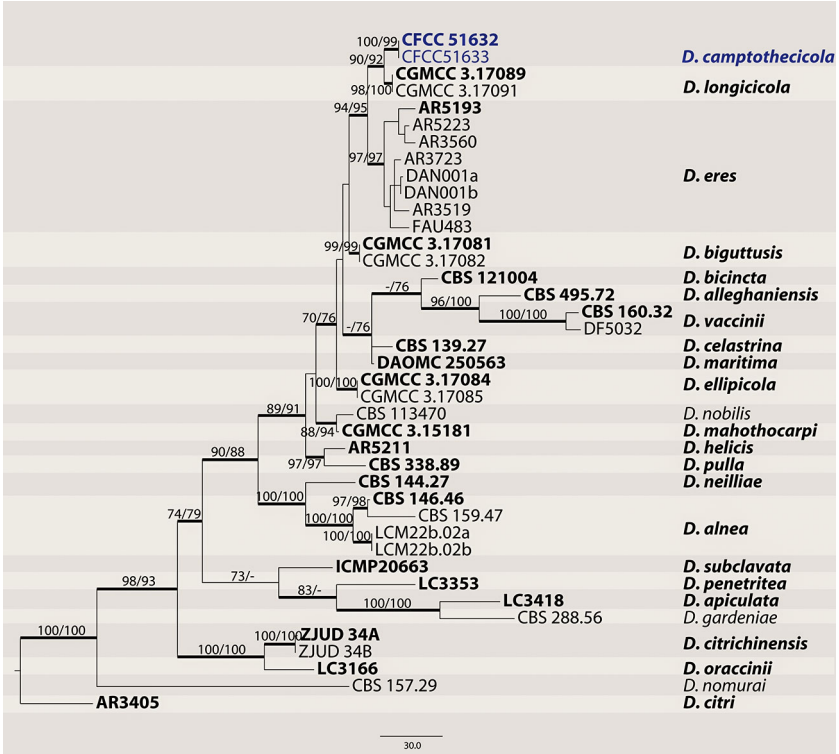

Plate 1. Phylogram of *Diaporthe* based on combined ITS, CAL, HIS, TEF1-α, and TUB2 dataset. MP and ML bootstrap support values above 70% are shown at the first and second position. Thickened branches represent posterior probabilities above 0.9 from BI. Scale bar = 30 nucleotide substitutions. Ex-type strains are in bold. Sequences generated in our study are in blue.

Donaldson 1995); the partial translation elongation factor 1-alpha (TEF1-α) region with primers EF-728F and EF-986R (Carbone & Kohn 1999); and the β-tubulin2 (TUB2) region with primers Bt2a & Bt2b (Glass & Donaldson 1995). The PCR amplification products were estimated visually by electrophoresis in 2% agarose gels. DNA sequencing was performed using an ABI PRISM® 3730XL DNA Analyzer with BigDye® Terminator Kit v.3.1 (Invitrogen) at the Shanghai Invitrogen Biological Technology Company Limited (Beijing, China).

DNA sequence analysis

The sequences of our isolates and the reference GenBank sequences (mostly those used by Udayanga et al. 2014b), are listed in Table 1; they were aligned using MAFFT v.6 (Katoh & Toh 2010) and edited manually using MEGA6 (Tamura et al. 2013). PAUP v.4.0b10 was used for maximum parsimony (MP) analysis (Swofford 2003); PhyML

v.7.2.8 for maximum likelihood (ML) analysis (Guindon et al. 2010); and MrBayes v.3.1.2 for Bayesian Inference (BI) (Ronquist & Huelsenbeck 2003). *Diaporthe citri* (AR3405) was selected as outgroup in this analysis (Udayanga et al. 2014b). Trees are shown using FigTree v.1.3.1 (Rambaut & Drummond 2010).

MP, ML, and BI analyses were applied to the combined ITS, CAL, HIS, TEF1-α, and TUB2 dataset. All characters were treated as unordered and equal weight. MP analysis was used, based on strict heuristic searches with a tree bisection and reconnection (TBR) algorithm. Maxtrees were set to 5000, branches of zero length were collapsed and all equally parsimonious trees were saved. Bootstrap values were calculated from 1000 replicates. Other calculated parsimony scores were tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency (RC). ML analysis was also performed with a GTR site substitution model (Guindon et al. 2010). The branch support was evaluated with a bootstrapping (BS) method of 1000 replicates (Hillis & Bull 1993). BI analysis with Markov Chain Monte Carlo (MCMC) algorithm was performed (Rannala & Yang 1996). The models of evolution were estimated by MrModeltest v.2.3 (Posada & Crandall 1998). Sequences were deposited in GenBank (Table 1), and the multilocus sequence alignment file was deposited in TreeBASE (www.treebase.org) as accession S20509.

Results

Molecular phylogeny

The combined (ITS, CAL, HIS, TEF1-α, TUB2) alignments for phylogenetic analysis contained 40 taxa (including one outgroup), comprising 2554 characters after alignment. Of these, 1929 characters were constant, 293 variable characters were parsimony-uninformative, and 332 characters were parsimony informative. The MP analysis resulted in 4 equally most parsimonious trees, with the first tree (TL = 1074, CI = 0.692, RI = 0.764, RC = 0.528) was shown in Plate 1. The phylogenetic tree obtained from ML and Bayesian analyses with the MCMC algorithm was consistent with the previous MP tree. The branches with significant Bayesian posterior probability (≥ 0.70) in Bayesian analyses were thickened in the phylogenetic tree. Isolates in the current study clustered in a distinct clade with a high support (MP/ML/BI=100/99/1) (Plate 1). The sequences were determined to represent a new species as described in this paper.

Taxonomy

Diaporthe camptothecicola C.M. Tian & Qin Yang, **sp. nov.** Plate 2

MycoBank MB 822351

Differs from *Diaporthe longicicola* by its smaller alpha and beta conidia, and shorter conidiophores.

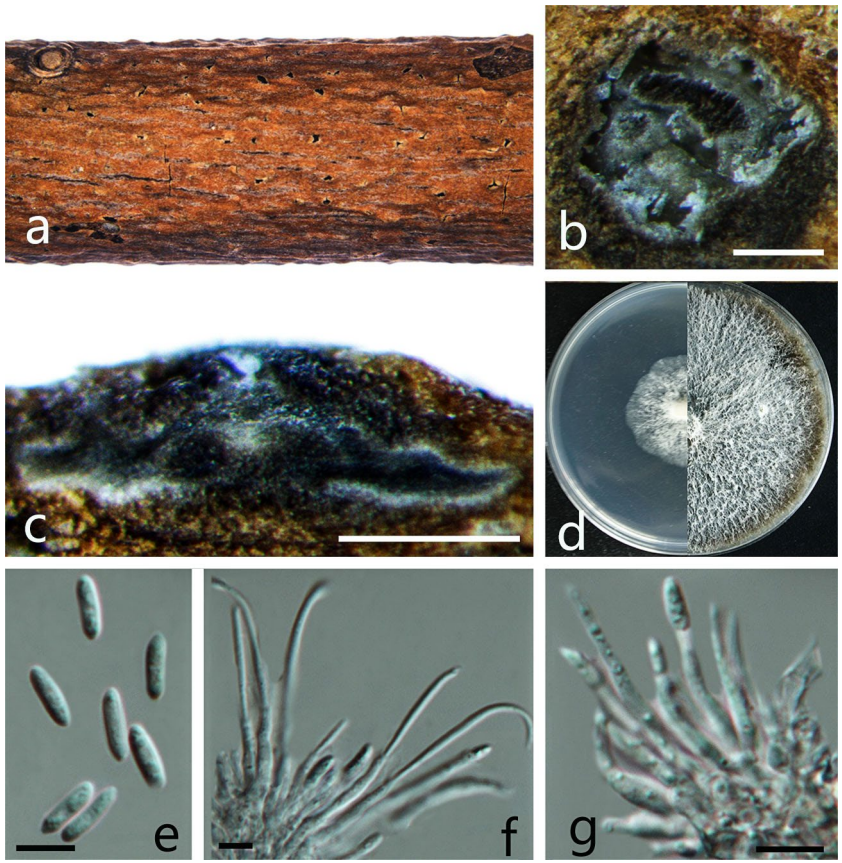

Plate 2. *Diaporthe camptothecicola* (holotype, BJFC-S1372) from *Camptotheca acuminata*. a: Habit of conidiomata on twig; b: Transverse sections through conidiomata; c: Longitudinal sections through conidiomata; d: Colonies on PDA at 3 days (left) and 30 days (right); e: Alpha conidia; f: Beta conidia; g: Conidiophores. Scale bars: b, c = 200 μm; e–g = 5 μm.

Type: China, Jiangsu Prov., Nanjing Agricultural University, 32°04′35″N 118°48′39″E, 9 m asl, on twigs and branches of *Camptotheca acuminata* Decne. (*Cornaceae*), 24 August 2014, coll. X.L. Fan (**Holotype,** BJFC-S1372; ex-type culture CFCC 51632).

Etymology: *camptothecicola* (Lat.), named after the host genus, *Camptotheca.*

Conidiomatal pycnidia immersed in bark, slightly erumpent through bark surface, sparse, globose to ovoid. Locule undivided, circular to ovoid, sometimes irregular, (450–)545–580(–600) μm diam. (av. = 560 μm, n = 20). Conidiophores (8.3–)12.5–15.8(–17.0) × 0.9–1.2 μm (av. = 13.2 × 1.1 μm, n = 50),

hyaline, unbranched, smooth, cylindrical, straight or slightly curved. Conidiogenous cells enteroblastic, phialidic. Alpha conidia hyaline, aseptate, oblong, inconspicuously biguttulate, (4.6–)5.5–7.0(–7.5) × 1.5–1.8 µm (av. = 6.2 × 1.6 µm, n = 50), more frequent than beta conidia. Beta conidia hyaline, aseptate, filiform or hamate with obtuse ends, (19.5–)23.3–27.5(–28.3) × 1.0 µm (av. = 25.1 × 1.0 µm, n = 50). Culture on PDA at first white, becoming grayish. Aerial mycelium white, cottony, with sparse, yellowish-brown and irregular margin, producing black pigment. Sexual morph not observed.

Host/Distribution: on branches of *Camptotheca acuminata* in China.

Additional specimen examined: **China, Jiangsu Prov.**, Nanjing Agricultural University, 32°04′35″N 118°48′39″E, 9 m asl, on twigs and branches of *Camptotheca acuminata*, 24 August 2014, coll. X.L. Fan (BJFC-S1373; living culture CFCC 51633).

Note: Two strains representing *D. camptothecicola* clustered in a well-supported clade, a species supported as distinct by molecular data. The most closely related species in the phylogram is *D. longicicola* Y.H. Gao & L. Cai. *Diaporthe longicicola* differs from *D. camptothecicola* by its larger alpha conidia (7.4 × 2.2 µm), its larger beta conidia (25–32.2 × 1.2–2 µm), and its longer conidiophores (14.1–22.5 µm; Gao et al. 2015).

Discussion

Phylogenetic analysis clustered the NAU collection within *Diaporthe* and closely related to *D. longicicola*. Both morphology and rDNA data support the Nanjing collection as a new species in the genus *Diaporthe*. Multi-locus gene regions were effectively used to identify *Diaporthe* species in this study as in many contemporary studies (Baumgartner et al. 2013; Huang et al. 2013; Tan et al. 2013; Dissanayake et al. 2015; Fan et al. 2015; Udayanga et al. 2014a, b, 2015; Gao et al. 2016).

Species recognition criteria in *Diaporthe* have historically been based on morphology and host affiliation. This has caused confused species delimitation (Aa et al. 1990, Rehner & Uecker 1994, Mostert et al. 2001, Santos & Phillips 2009, Udayanga et al. 2012a). A *Diaporthe* species previously reported on this host, *Phomopsis camptothecae* C.Q. Chang et al., was determined to be a synonym of *Diaporthe sojae* Lehman (Udayanga et al. 2015). *Diaporthe camptothecicola* can be distinguished by its conidiophores and alpha conidia; *D. sojae* has wider alpha conidia (5.3–7.3 × 2–3 µm) and wider conidiophores (2–4 µm; Udayanga et al. 2015). Phylogenetic analysis based on multi-locus (ITS, CAL, HIS, TEF1-α, and TUB2) demonstrated the distinctive new species in *Diaporthe* with highly supported clades (MP/ML/BI = 100/100/1).

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